TITLE: Elevated MMP-9 in the Thoracic and Lumbar Cord Impedes Activity Dependent Plasticity and Recovery Early after SCI in Mice.

AUTHORS: Christopher N. Hansen[1], Rochelle Deibert[2], Lesley C. Fisher[2], Lyn Jakeman, Ph.D[1,3], Haoqian Zhang, Ph.D[4], Linda Noble-Haeusslein, Ph.D[4], and D. Michele Basso, PT, Ed.D*[1,2]

1 Neuroscience Graduate Studies Program, The Ohio State University, Columbus, OH

2 School of Health and Rehabilitation Sciences, The Ohio State University, Columbus, OH

3 Department of Physiology and Cell Biology, The Ohio State University, Columbus, OH

4 Departments of Neurological Surgery and Physical Therapy and Rehabilitation Science, University of California, San Francisco, CA

1. Introduction

A loss in mobility is one of the most noticeable and debilitating consequences of spinal cord injury (SCI). Activity-dependent tasks like treadmill (TM) training harness endogenous spinal plasticity to promote motor relearning and recovery (Hodgson et al., 1994), (Leblond et al., 2003), (Basso and Hansen, 2011). Despite modest improvements with TM training in the clinic, deficits persist and complete recovery is unattainable for most individuals (Buehner et al., 2012), (Harkema et al., 2012). Optimal recovery may not only depend on the training task, but when it is administered after injury and its primary site of action in the central nervous system (CNS). Indeed, early CNS injury induces a robust period of plasticity comprised of structural and synaptic changes throughout the neuroaxis (Fawcett, 2009). Delivering locomotor training when plasticity is primed has the potential to produce greater functional improvement. Surprisingly,

some forms of exercise have proven detrimental and even disrupt neurovascular integrity if delivered too early (Smith et al., 2009), (Caudle et al., 2011), (Kozlowski et al., 1996), (Maldonado et al., 2008), (Griesbach et al., 2007). Whether TM training directed at lumbar neural networks can be optimized by delivering it early or late after contusion injury, remains unexamined. Likewise, potential cellular and molecular factors mediating task-dependent training have only recently been identified remote to the primary SCI site (Detloff et al., 2008), (Nesic et al., 2005), (Hains and Waxman, 2006), (Andrews et al., 2012). Neuroinflammation is a known impediment to spinal learning and plasticity (Yirmiya and Goshen, 2011), (Vichaya et al., 2009), (Huie et al., 2012). Beyond lesion-site pathology, an important aspect of activity-dependent plasticity is the cellular and molecular microenvironment of the lumbar enlargement, which is largely intact and responsive to sensorimotor cues. We previously showed that activated microglia and cytokine expression extend at least 10 segments caudal to the lesion in the lumbar enlargement and contribute to sensory dysfunction (Detloff et al., 2008). Remote inflammation, alongside changes in extracellular matrix composition (Andrews et al., 2012) may disrupt synaptic efficacy and impede plasticity in locomotor interneuron networks. Matrix metalloproteinases (MMPs) are a potential mediator of these effects as they regulate diverse functions, including tissue remodeling, inflammation, and learning (Ethell and Ethell, 2007), (Zhang et al., 2011), (Parks et al., 2004), (Vu et al., 1998). In particular, the gelatinase MMP-9 strongly promotes blood spinal cord barrier (BSCB) permeability and proinflammatory cytokine production in the CNS (Noble et al., 2002), (Kawasaki et al., 2008). MMP-9 is produced by various cell types including trafficking leukocytes, resident glia, and vascular endothelia and shows a similar expression profile in rodent and human SCI (Zhang et al., 2011), (Buss et al., 2007). Whether MMP-9 is expressed

remote to the SCI is unknown. If MMP-9 is produced in remote lumbar regions, neurovascular reactivity may impede plasticity and recovery of function even when TM training is delivered. Therefore, the purpose of the study was to determine if remote production of MMP-9 occurs after T9 SCI and influences motor relearning and recovery. We present novel findings of MMP-9 regulated inflammation in the lumbar enlargement during the first week after SCI in C57BL/6 (WT) mice. To determine if an optimal period of locomotor plasticity occurs after SCI that depends on MMP-9, we delivered lumbar-focused treadmill (TM) training interventions in WT and MMP-9 null mice early after SCI. Robust training-induced recovery occurred in the null mice. In WT mice, the same early intervention resulted in significant locomotor deficits and suggests neurotoxic effects. This work has been presented in abstract form (Hansen CN, 2012); (Hansen CN, 2011).

2. Materials and Methods

2.1 Subjects and surgeries

Experiments were conducted in accordance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Adult (3-4 months of age) female MMP-9 null (FVB/NJ; (Vu et al., 1998), (Oh et al., 1999) and C57BL/6 wild-type (WT) mice were obtained from Jackson Laboratories. The MMP-9 null mouse shows a mild delay in bone formation (Vu et al., 1998), which was accounted for in kinematic assessments by collecting actual femur and tibia bone lengths at the time of sacrifice. Laminectomy and spinal cord contusions were performed as described previously (Jakeman et al., 2000), (Kigerl et al., 2006). Briefly, mice were anesthetized with a ketamine (80 mg/kg)-xylazine (20 mg/kg) cocktail and given prophylactic antibiotics (gentocin, 1mg/kg). Using aseptic techniques, removal of the spinous

process and lamina of T9 exposed the dura. After stabilizing the vertebral column, the Infinite Horizon (IH) device delivered 75 kilodynes of force to induce a severe contusion injury. Biomechanics of the injury were screened on day 0, and outlier displacements were excluded (n=8). The incision was closed in layers and 2cc of sterile saline was given subcutaneously to prevent dehydration. During recovery, mice received antibiotics (5mg/kg gentocin, s.q.) and saline for 5 days and bladders were manually expressed twice per day until tissue harvest (Hoschouer et al., 2010). To ensure injury severity, BMS scores were screened on day 1 to be less than 0.5 for training studies, and less than 3 for ELISA or histology studies. Any behavioral outliers that scored 3 or above on the BMS at 1d were excluded from the study (n=4).

2.2 Protein isolation and quantification

In trained and untrained WT and MMP-9 null mice (n=62), fresh tissue was harvested from the lumbar enlargement (Naïve, 1, 2, 3, 7, 9 and 42d post-injury) to characterize cytokine and MMP expression and activity. Mice were perfused with sterile saline, and spinal cords were quickly dissected, snap frozen, and stored at -80°C. Segments from L1-3 were blocked and cut in 20um cryostat sections for in situ gelatinase zymography. Segments from L4-5 were homogenized in RIPA lysis buffer (Pierce) and protease inhibitor cocktail (Roche). After centrifugation at 10,000g for 5 min, protein concentrations were determined by a BSA protein assay. Quantification of matrix and cytokine proteins was determined using a custom SearchLight Multiplex ELISA Array and performed by Aushon biosystems. The custom-made arrays were produced by spotting with capture antibodies specific to tumor necrosis factor (TNF)-α, interleukin (IL)-1β, MMP-2, MMP-3, and MMP-9. The bound proteins were then detected with a biotinylated detection antibody, followed by streptavidin-horseradish peroxidase (HRP) then were visualized with SuperSignal ELISA Femto Chemiluminescent substrate. The luminescent

signal produced from the HRP-catalyzed oxidation of the substrate was measured using the SearchLight Imaging System (Pierce) and protein concentrations extrapolated from a standard curve using ArrayVision (Pierce).

2.3 In situ gelatinase zymography

In situ zymography was used to detect and localize gelatinase activity in fresh tissue sections (Oh et al., 1999), (Noble et al., 2002), (Goussev et al., 2003). Sections were incubated in 0.05 M Tris-HCL, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mM NaN3, pH 7.6, containing 40 ug of FITC-labeled gelatin (Molecular Probes, Eugene, OR), at 37°C for 1 hour. The gelatin is tagged to a peptide that fluoresces when cleaved by gelatinolytic activity. This reaction was then visualized with fluorescent microscopy. Because this assay localizes gelatinase activity but does not distinguish between MMP-2 and 9, we performed the in situ stain in the same mice used for ELISA. Sections were imaged using a Nikon Elipse E800 Microscope for fluorescence.

2.4 Histology

Mice (n=18) were transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (pH 7.4) at 1 or 7 days post injury (dpi). Spinal cord segments at the T9 lesion site (10mm) and from L1-L5 were post-fixed for 1h in 4% paraformaldehyde, rinsed overnight in 0.2M phosphate buffer (PB, pH 7.4) then cryoprotected in 30% sucrose before being frozen on dry ice (Detloff et al., 2008), (Ma et al., 2004;Ma et al., 2001),(Basso et al., 2006). Both epicenter and lumbar blocks were sectioned and collected in series of equally spaced sections at 20 um on a Microm HM505E cryostat and placed on ColorFrost Plus slides (Fisher Scientific).

Fluorescent immunohistochemistry was performed on transverse spinal cord sections of lumbar segments. A 1:200 dilution was used for Rabbit anti Iba-1 (Wako). Antibodies were prepared in

blocking solution containing 1%BSA/0.1%FG/3%NGS/0.2%Tx-100 in PBS. Incubation of primary antibodies occurred overnight at 4°C. Secondary antibodies were used at a 1:400 dilution. Final detection of signal was visualized under fluorescence. For all staining, control sections were processed by eliminating the primary antibody and replacing with blocking solution to ensure positive labeling. Sections were imaged using a Zeiss 510 Laser Confocal Microscope (The Ohio State University Confocal Microscopy Imaging Facility (CMIF)) for fluorescence.

The lesion site was transversely sectioned (20um) and stained for myelin using eriochrome cyanine. The section with the largest lesion and least amount of stained white matter represented the lesion epicenter. Area of stained white matter at the epicenter was divided by the total cross sectional area to serve as a measure of injury severity (Kloos et al., 2005).

2.5 Training paradigm

Treadmill (TM) training was delivered in subsets of mice at early (2 - 9d; ETM; n= 12 KO, n=11 WT) timepoints alongside untrained controls (n=13 KO, and n=10 WT). An independent group of Naïve controls (n=10) were used for kinematic comparisons. Training consisted of eight consecutive days of manually delivered weight supported stepping during quadrapedal locomotion on a custom-built TM (Columbus Instruments). Each session included two 10-minute bouts separated by a 20-minute rest interval to prevent delayed onset muscle soreness (McHugh, 2003). Adjustable harnesses provided body weight support while maintaining the trunk in a typical horizontal murine posture (Figure 5). Manually-assisted HL stepping was provided as needed using small rounded pestles to achieve toe clearance and plantar placement of the paw on the TM belt.

2.6 Locomotor Assessments

Open Field

Locomotor recovery was assessed using the 9-point Basso Mouse Scale (BMS) for locomotion by two raters blind to group assignment (Basso et al., 2006). Open field activity of each mouse occurred for 4 minutes prior to injury, at 1, 2, 3, 4 and 7 dpo, and weekly thereafter. Rating criteria considered joint movement, weight support, plantar stepping, coordination, paw position, as well as trunk and tail control. Scores range from 0-9 representing no HL movement (0) to normal locomotor function (9).

Two-Dimensional Kinematics

All mice had two-dimensional (2D) kinematic recordings of TM walking using a novel application for mice based on established criteria for rat and feline models (Hansen et al., 2012), (Basso et al., 1994). Kinematic assessments were done prior to injury (naïve) and again at 7, 35, and 42dpo. Retention of training effects was determined in ETM groups by assessing locomotor function 4 weeks after training ended (35d). To demarcate appropriate hindlimb (HL) joints, naïve mice were briefly anesthetized with 2% isofluorane in air to identify joint location. While unconscious, the left HLs were shaved and bony prominences palpated and marked. Appropriate marker placement was later confirmed during locomotion. We used Nair hair remover (©Church and Dwight) to maintain marker locations for the study duration. Marked prominences included the iliac crest, greater trochanter, femoral condyle, lateral malleolus, and head of the fifth metatarsal (Figure 5). A videotaped record of quadrupedal TM locomotion was collected using a Panasonic WV-CL350 camera (60Hz) with a time-code generator. Kinematic markers were digitized using PEAK Motus. To account for movement of the knee joint during locomotion, a triangulation program was used to estimate its position (Goslow, Jr. et al., 1973)

Actual femur and tibia bone lengths were collected at sacrifice and used with the hip and ankle X, Y positions to derive location. Only animals that were able to produce a minimum of 18 plantar steps on the TM were used for kinematic analyses. Dorsal stepping and non-stepping animals were excluded and statistically penalized for nonperformance.

Two-dimensional (2D) treadmill and gridwalk kinematics were used to examine discrete components of locomotion. Total time in swing (lift off-initial contact) and peak velocity of the toe and ankle joints were determined from video frame analysis. Trunk instability was determined by calculating maximal vertical displacement of the pelvis during stepping. Toe dragging was measured by comparing the times of initial forward movement of the toe and visual confirmation of lift off. These assessments were averaged over 20 steps.

Gridwalk

A 2.54cm square metal grid apparatus was used to measure precise paw placement (Ma et al., 2001). Mice were gentled to the grid pre-operatively, and then tested at 35 and 42d. Volitional movement occurred for 5 forward-progressive bouts. Success was determined by a weight-supported step from rung to rung. The number of HL misses are expressed relative to the number of forelimb steps and shown as percent success.

2.7 Statistics

All behavioral outcome measures were analyzed using MANOVA (ETM) or repeated measures (LTM) ANOVA with Dunnetts or Tukey's post hoc tests. Means and standard error of the mean (SEM) are reported throughout. For kinematic data collection, not all mice were able to perform the task (ie dragged or dorsal stepped). Nonperformance was corrected by assigning the maximal kinematic value plus one standard deviation from the mean. Group comparisons for

open field plantar stepping and weight support were made using a Chi square analysis. Protein levels are displayed as percent naïve and were analyzed using a one-way ANOVA. When appropriate, differences between protein measures were evaluated with a students *t*-test. Correlations between white matter sparing and proportional area of iba1-labeling were made using Pearson's correlation analysis.

3. Results

3.1 Acute injury mechanisms extend to the lumbar enlargement after thoracic SCI.

Thoracic spinal cord contusion results in increased cytokine and metalloproteinase expression in the lumbar enlargement (L4/5) during acute stages following injury (Figure 1). Within 24h, pro-gelatinase MMP-3 protein was significantly elevated (423.9% Naïve +/- 105.9; $p<0.0001$) and returned to basal levels by 2d. MMP-9 expression reached a more than 5-fold increase at 7d and persisted at 9d (526.4% Naïve +/- 73.3 and 547.2%Naive +/- 144.1 respectively; $p<0.0001$). Gelatin zymograms confirm expression of pro-MMP-9 in both trained and untrained WT at 9d (Figure 6).

In situ gelatinase zymography localized MMP activity in tissue sections from L1-3 blocks of the same lumbar spinal cords. In the uninjured spinal cord, low gelatinase activity occurred in the meninges and sparse labeling of vasculature corresponding to locations reported previously (Figure 1h; (Noble et al., 2002), (Goussev et al., 2003). By 3d after SCI, a pronounced increase in gelatinase activity is evident in vascular structures that remains elevated at 7 and 9d. The number of branches and width of vessels labeled with gelatinase activity was greatest in the lumbar cord at 3dpi (Mean width: 14.5um, mean branches: 5.33; $p<0.05$ compared to Naive). In

addition, enhanced gelatinase-positive cells were evident in dorsal and ventral roots at 7 and 9d (Figure 1g).

WT mice showed robust microglial activation in the lumbar enlargement after thoracic SCI. Within 24h, iba1-labeled microglia displayed significantly larger cell bodies (mean width: 19.2um +/-1.73; $p<0.0001$) and a bushy phenotype (Figure 2; similar to phenotypes described by Kumar et al. 2012). By 7d, pronounced microglial activation remained with a hypertrophic phenotype compared to finely ramified microglia in naïve controls (mean width 14.1um +/- 1.08; $p<0.05$). Increased microglial activation occurred throughout sectional gray matter lamina and funniculi of the white matter from L1-2 (mean area: 1.8% +/- 0.51 area in naïve and 8.03% +/- 0.78 area in 7d WT $p<0.05$). The greatest microglial density occurred in the dorsal horn relative to the ventral horn after SCI. Changes in microglia phenotype occurred alongside a rise in pro-inflammatory cytokine production. Protein expression of the pro-inflammatory cytokine, TNFa, reached a nearly 2-fold increase by 9d (162% +/- 86.3 Naïve; $p<0.05$), while levels of IL-1β and MMP-2 remained at baseline in the lumbar enlargement during the first week after SCI.

3.2 Inhibition of MMP-9 results in white matter sparing and reduces inflammation in the lumbar enlargement

Similar to findings of Noble and colleagues (2002) in a moderate injury model, we show that removal of MMP-9 results in modest white matter sparing at the lesion site (Noble et al., 2002). After severe SCI, lesion size was smaller (78.8% +/- 1.49 Area in KO, and 87.06% +/-2.32 in WT; $p<0.05$) and the amount of white matter sparing was significantly greater in KO mice (21.1% +/- 1.49 WMS in KO, and 12.93% +/- 2.33 in WT; $p<0.05$). White matter sparing at the lesion site predicted the extent of microglial activation in the lumbar enlargement. At 7d, mice

with greater sparing at T9 had significantly less cross-sectional iba-1 labeling throughout L1/L2 (r^2=0.6745; p<0.01).

Pro-gelatinase MMP-3 maintained an acute response in KO mice and reached an almost 4-fold increased within 24h (386.5% Naïve +/- 57.9; p<0.05) but returned to baseline by 3d. MMP-2 levels remained at baseline in WT mice at 42d, and did not show a compensatory increase in MMP-9 null mice (mean MMP-2 expression in KO: 118.8% Naïve +/- 18.7, WT: 105.6%Naive +/-8.9; p=.71). Microglial activation was markedly reduced in MMP-9 KO mice compared to WT at 7d (Figure 2). In both the dorsal and ventral horn, microglia maintained a hypertrophic phenotype but over much less area (328.4% reduction in dorsal horn, 193.4% reduction in ventral horn; p<0.05; Figure 3). Deletion of MMP-9 reduced pro-inflammatory cytokine production in the lumbar enlargement over the first week after SCI. In WT mice, TNFa expression reached an over 2-fold increased by 7d (Figure 4; 222.4% Naïve +/- 86.4; p<0.05). In MMP-9 null mice, TNFa expression was significantly reduced in L4/5 at 24h (52% reduction relative to naïve; Figure 4b; p<0.05). At 7d, deletion of MMP-9 restored TNFa levels to homeostatic levels (79.7% Naïve +/-15.09; p<0.05 compared to naive). IL-1b remained at baseline levels in L4/5 at all timepoints.

3.3 Early TM training promotes robust recovery only in MMP-9 null mice

Severe SCI resulted in paresis during overground locomotion with only occasional, slight HL movements at 24h in all groups (Mean BMS=0.27 +/- 0.05 KO, 0.26 +/-0.07 WT). Early TM training delivered 2-9dpi (ETM) resulted in substantial locomotor improvements by 7d in MMP-9 KO but not untrained KO or trained or untrained WT groups. At 7d, recovery was negligible and failure to demonstrate weight supported stepping occurred in 100% of trained WT mice, 80% of untrained WT mice and 70% of untrained KO mice (Figure 6a). Plantar stepping

occurred in 66.7% of ETM KO mice (Mean BMS=3.83+/-0.32). This was similarly observed during treadmill locomotion, as trained MMP-9 KO mice were the only group capable of generating kinematic metrics (Figure 6b). Untrained KO and trained and untrained WT groups required an assistive harness for bodyweight support and consistently dragged HLs on the TM. Neither training alone nor MMP-9 depletion alone promoted recovery.

Functional improvements in ETMKO outlasted the training period by 4 weeks. Retention was examined at 35d using state of the art TM kinematics and motor control assessments on gridwalk (Figure 7). Groups did not differ in open field recovery at 35d (Mean BMS= 5.56 +/- 0.17 ETM KO, 5.56 +/- 0.25 Unex KO, 4.58 +/- 0.55 ETM WT, and 5.05 +/- 0.24 Unex WT). Kinematic metrics identified significant differences in TM performance. At 35d, trained MMP-9 null mice stepped with near-normal toe and ankle velocity during TM walking (Mean 27.79 +/- 2.02 and 19.96+/- 1.9 cm/sec respectively) while all other groups were significantly slower than Naïve ($p<0.05$). Measures of toe dragging were markedly reduced in trained MMP-9 KO mice and not statistically different from Naïve (Mean toe Drag: 0.0407+/- 0.003 seconds). In contrast, early training of WT mice resulted in significant locomotor deficits. During swing on the TM, WT mice showed notable reductions in velocity in both the toe and ankle (Mean Velocity: 4.20cm/sec and 3.33cm/sec respectively; $p<0.05$). Measures of trunk instability, toe dragging, ankle velocities, and swing time were significantly worse than Naïve in trained WT($p<0.05$). On the gridwalk, early training resulted in significantly worse stepping precision. Weight supported, rung-to-rung stepping was only successful in 5.49% +/- 2.54 of attempts in WT, while unexercised WT stepped with a 13.58% +/- 1.81 success rate.

4. Discussion

Spinal cord injury creates dual and conflicting cellular processes along the neuraxis. Mechanisms of both plasticity and inflammation occur remote to the lesion in the lumbar enlargement that influences function (Detloff et al., 2008), (Andrews et al., 2012), (Hains and Waxman, 2006), (Hougland et al., 2012). Careful delineation of these processes is required, as future SCI therapeutics will likely accompany lumbar-focused interventions through rehabilitation. We show for the first time that elevated MMP-9 produces an inflammatory microenvironment at substantial distances from the lesion epicenter during acute SCI. Removal of MMP-9 reduces lumbar inflammation and allows robust locomotor plasticity and recovery but only with an early intervention. Our work demonstrates a negative, time-dependent interaction between lumbar MMP-9 production and motor relearning.

MMP members display a distinct temporal progression in the lumbar enlargement during the first week after SCI. Cell types including resident glia, trafficking leukocytes, and vascular endothelia produce MMPs in CNS trauma (Zhang et al., 2011). Pro-gelatinase MMP-3 shows a rapid upregulation within 24h that precedes MMP-9 at 3, 7, and 9d (Figure 1). A similar trend is observed in the brain when a select entorhinal cortex lesion results in distant expression of MMP-3 that facilitates synaptogenesis and remodeling in the dentate gyrus (Kim et al., 2005a), (Falo et al., 2006). MMP-3 is considered to be the primary activator of MMP-9 and likely plays a critical role in the transcription and activation of MMP-9 at later time points in the lumbar cord (Vempati et al., 2007). In situ and gelatin zymograms confirm MMP-9 expression and localize activity to microvascular endothelia in lumbar segments up to 9 days after SCI (Figure 1h). Our findings of gelatinase activity around BSCB constituents may suggest permeability or neurovascular reactivity. It is possible that elevated MMP-9 could result in part from barrier

disruption since gray matter permeability extends to at least L1-L2 in rat models of midthoracic contusion (Popovich et al., 1996).

Midthoracic contusion produced a rapid, pro-inflammatory shift in resident microglia around lumbar CPGs. At 24 hr during peak MMP-3 expression, microglia displayed an exaggerated activation profile, or bushy phenotype that may indicate a pre-migratory state (Kumar et al., 2013). In culture, MMP-3 functions as a novel signaling protein to activate microglia and may be one of the earliest microglial regulators remote to the injury site (Kim et al., 2005b). Hypertrophic microglia were evident throughout dorsal and ventral regions of the lumbar cord throughout the first week after SCI, reaching significance at 7d (Figure 2). The prolonged increase in hypertrophic phenotype may be part of a paracrine/autocrine loop in which MMP-9 from endothelial cells regulates pro-inflammatory cytokine availability and MMP-9 production in microglia via NFkB and p38 signaling as shown in other cell types (Rajashekhar et al., 2011). Reduction of iba1-labeling in MMP-9 null mice may reflect MMP-9 dependent migration, as glial (astrocyte) migration is markedly reduced in MMP-9 null mice after SCI (Hsu et al., 2008). Additionally, the possibility exists that MMP-9 has an indirect influence on lumbar microglia by regulating axonal dieback and demyelination at the injury site (Busch et al., 2009); (Liu and Shubayev, 2011). Regardless of the downstream actuators, we show lumbar microglial regulation is MMP-9 dependent given that elimination of MMP-9 restored microglia to a resting phenotype and normal levels in the lumbar cord (Figure 2). Consequently, expression of TNFa was significantly reduced at 24h and restored to homeostatic levels by 7d in MMP-9 null mice (Figure 4).

We show for the first time that MMP-9 dependent factors interact negatively with TM training to impede locomotor recovery. The pro-inflammatory microenvironment created early

after midthoracic contusion appears to limit efficacy of TM training. By eliminating MMP-9 in combination with early training, we restored consistent stepping by 7 days – an extent of recovery unattained by training alone or removal of MMP-9 alone. Moreover, these training effects extended 4 wks after the intervention ended. In our activity-based training paradigm, repetitive sensorimotor cues are delivered to the lumbar enlargement to facilitate oscillatory motor output from lumbar CPGs. Training produces synaptic strengthening and reorganization within interneuronal networks to promote locomotor recovery, a process collectively termed motor relearning (Hodgson et al., 1994), (Rossignol et al., 2006), (Cazalets et al., 1996). In these lumbar systems, neuroinflammation limits synaptic formation and long term potentiation, two necessary features of motor relearning (Yirmiya and Goshen, 2011); (Vichaya et al., 2009), (Young et al., 2007). We propose that MMP-9 creates an inhibitory microenvironment in the lumbar cord and, as in isolated assessments of instrumental learning, the resulting microglial activation and pro-inflammatory cytokine production prevents motor relearning and recovery (Vichaya et al., 2009), (Huie et al., 2012),(Young et al., 2007).

Surprisingly, white matter sparing by itself was insufficient to produce functional gains in our model of severe SCI. Untrained MMP-9 null mice did not differ from WT in the open field (Figure 6) which contrasts with the improved locomotor recovery reported by Noble and colleagues after reducing or eliminating MMP-9 in a less severe injury (Noble et al., 2002). We propose that modest white matter sparing is likely too small to support the robust recovery attained with training in MMP-9 null mice. Rather, changes at the epicenter may have influenced lumbar neuroinflammation since we found a relationship between sparing and lumbar microglial activation (Figure 3C). Thus, attenuation of inflammation may be a necessary first step in inducing activity-dependent plasticity of segmental and spared descending systems.

Our findings may suggest a critical period of spontaneous plasticity acutely after SCI that offers a novel opportunity to influence locomotor recovery. In our mouse model, we show that this period is defined by a period of days. However, marked differences in the onset and duration of inflammation in human SCI suggests that the optimal window to modify neuroinflammation and create a permissive environment for rehabilitation clinically may be weeks or months after the initial injury (Donnelly and Popovich, 2008), (Fleming et al., 2006).

An important finding was that intervening too early after SCI with TM training worsens function more than had no exercise been applied. In WT mice, we found that early TM training in a nonpermissive microenvironment produced greater deficits gridwalk and in locomotor kinematics than untrained SCI alone (Figure 7). These findings are consistent with work in SCI and other CNS injury models (Jones and Schallert, 1994), (Kozlowski et al., 1996), (Smith et al., 2009), (Caudle et al., 2011). Early after SCI, increasing vascular demand when BSCB integrity is compromised may exacerbate pathology. In fact, a single session of swim training delivered in acute stages of SCI increases lesion-site permeability (Smith et al., 2009). Whether MMP-9 facilitates exercise-mediated disruption of BSCB components is unknown. This is likely, considering that acute phase BSCB breakdown is reduced in MMP-9 null mice (Noble et al., 2002). In our model, increased expression of MMP-9 persists in lumbar segments of both trained and untrained WT mice (Figure 6). Mitigation of remote neurovascular reactivity in MMP-9 null mice likely facilitated plasticity and recovery in the ETM paradigm. Late after SCI when reconstitution of the BSCB is evident, MMP-9 inhibition may not facilitate lumbar plasticity (Whetstone et al., 2003), (Popovich et al., 1996). Together, our work shows that applying an effective treatment too early after injury produces greater motor impairments than if no treatment had been delivered.

The extent of remote injury mechanisms that occur in the lumbar enlargement after thoracic SCI identifies our limited knowledge of post-injury dynamics. Disruption of neurovascular interactions around locomotor CPGs results in profound functional consequences. We provide clear evidence that delivering locomotor training is ineffective and potentially neurotoxic early after SCI. If delivered at the correct time and in a permissive lumbar microenvironment, locomotor training results in robust recovery. Intervention timing provides a novel opportunity to influence plasticity and we show that remote production of MMP-9 is a clear impediment. Combinatorial therapies that reduce inflammation to permit synaptic and structural remodeling in the lumbar enlargement will likely promote the most robust functional gains. The future of neurorehabilitation after SCI must consider at- and below-level injury effects that may jeopardize the recovery of locomotion.

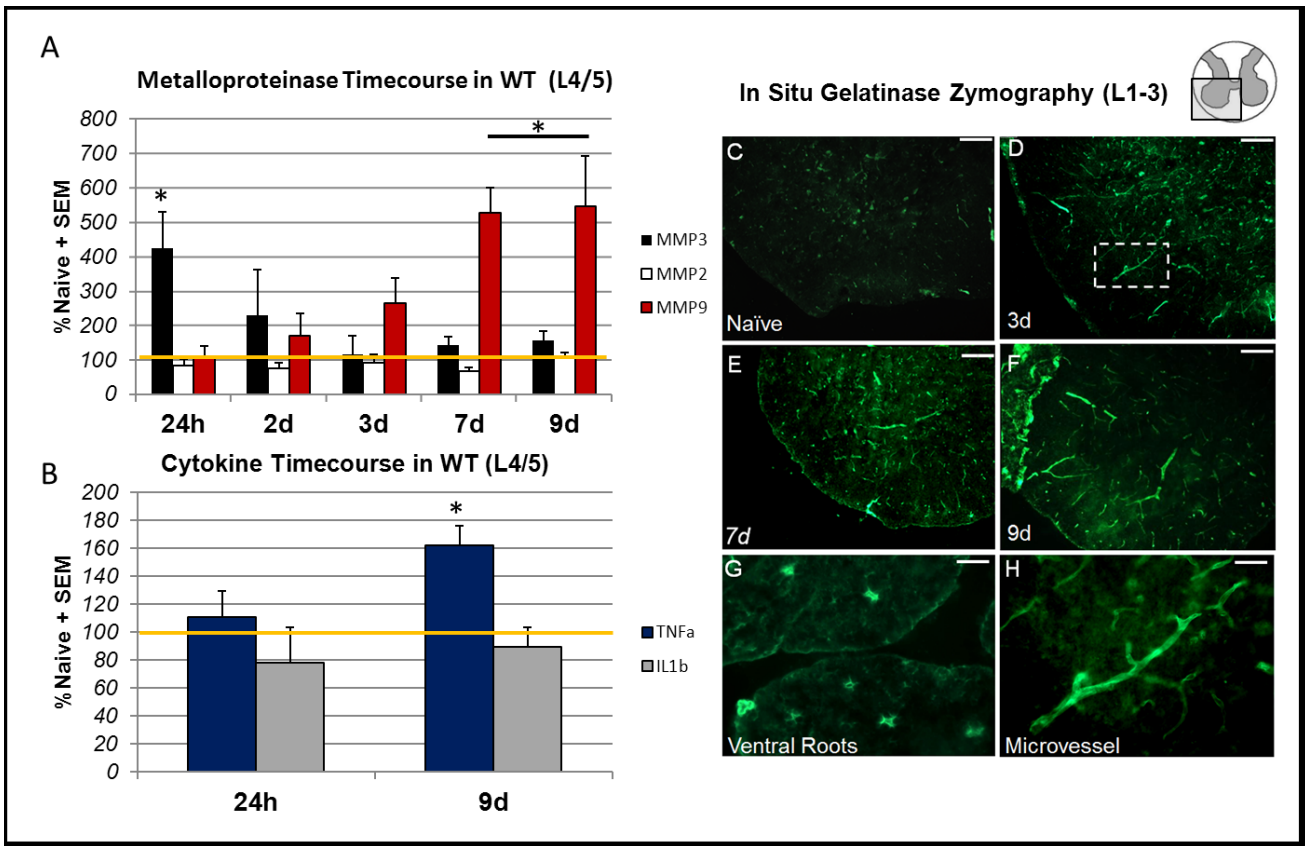

Figure 1. *Remote injury mechanisms extend to the lumbar enlargement during acute stages of SCI.* Protein analyses reveal increased metalloproteinase and cytokine expression in the lumbar enlargement (L4/5) over the first week after SCI (n=38). a) Within 24 hours, pro-gelatinase MMP-3 was significantly upregulated (423.9%; $p<0.0001$ compared to Naive) but returned to baseline by 9d. MMP-9 reached peak elevation at 9d (547.2%; $p<0.0001$ compared to Naive). MMP-2 protein remained at baseline. b) Pro-inflammatory TNFa reached a nearly 2-fold increase at 9d (162.1%; $p<0.002$ compared to Naive). In situ gelatinase zymography localized MMP gelatinase activity around microvascular endothelia in lumbar segments. Sparse labeling of vasculature is evident at in the naïve cord (c). After SCI, more pronounced gelatinase activity is evident around vascular endothelia by 3d (d,f) and remains evident at 7 and 9d (g,h). Vascular

structures are notably wider and display more branching at 3d compared to naive (Mean 3d:14.5 micron, 5.33 branches; Naïve: 6.2 micron, 1.1 branches $p<0.05$).

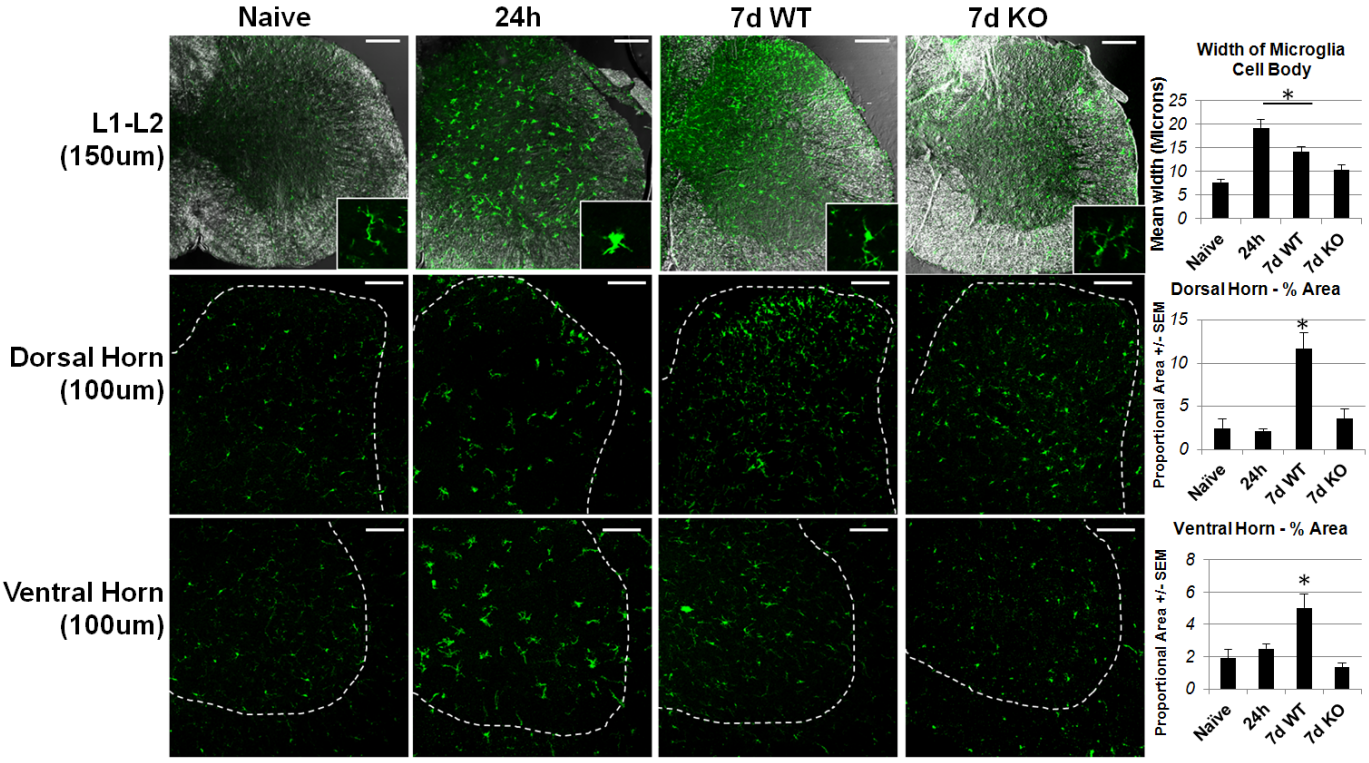

Figure 2. *Thoracic SCI results in rapid activation of microglia in the lumbar enlargement.* Within 24h, iba1 labeling shows that resident microglia display an activated bushy phenotype made evident by increased cell body width (mean 19.25um; $p<0.001$ compared to naive). By 7d in WT mice, microglia maintain hypertrophy and cover greater proportional area in the dorsal horn (11.6% area; $p<0.01$ compared to naïve, 24h WT, and 7d KO) and ventral horn (5.02% area; $p<0.05$ compared to naïve, 24h WT, and 7d KO). Deletion of MMP-9 resulted in a significant attenuation of microglia phenotype and proportional area. MMP-9 null mice showed reduced cell body width (10.27um; $p<0.01$ compared to 24h) and reduced labeling in dorsal horn

(3.54% area; $p<0.001$ compared to 7d WT) and ventral horn (1.35% area; $p<0.01$ compared to 7d WT).

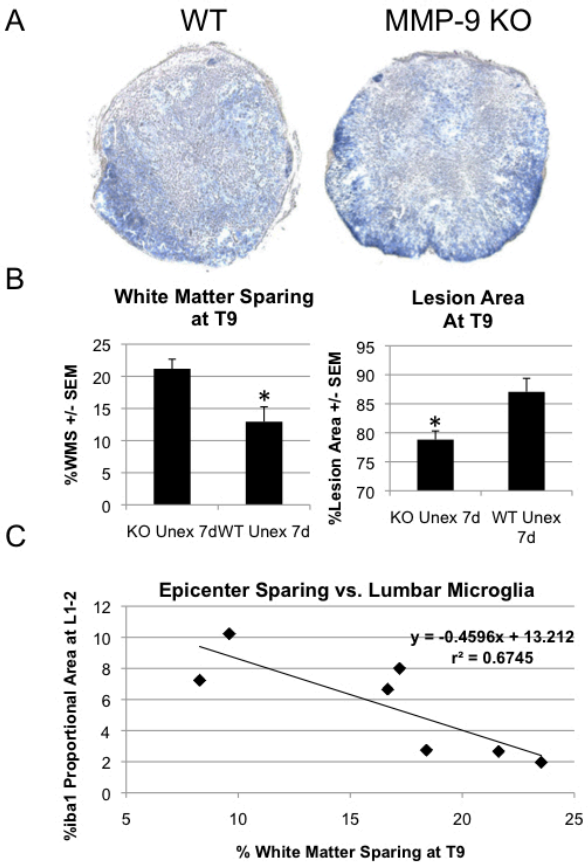

Figure 3. *Deletion of MMP-9 results in white matter sparing that predicts remote activation of microglia.* Representative epicenters are shown from KO and WT mice stained for myelin after severe SCI (A). MMP-9 KO mice had notably greater myelin sparing (B; 21.1% KO, 12.9% WT; $p<0.05$) and smaller lesion size (B;78.8% KO, 87.1% WT; $p<0.05$). The extent of white matter sparing at the lesion epicenter was highly predictive of remote microglial activation. Greater white matter sparing at T9 resulted in less iba1-labeling at L1-2 throughout the entire cross section (C; $r^2=0.6745$; $p<0.05$ Pearson's correlation).

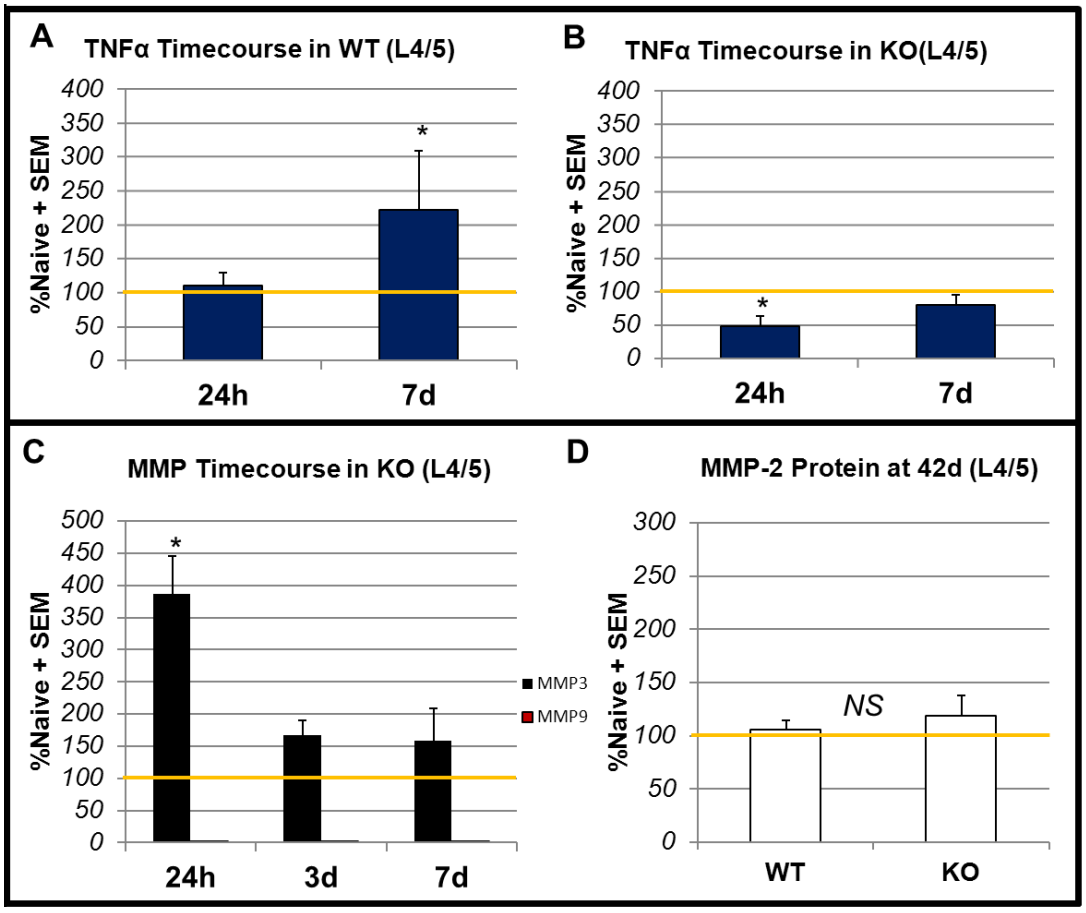

Figure 4. *Deletion of MMP-9 attenuates pro-inflammatory cytokine but not metalloproteinase expression.* By 7d, increased expression of TNFa protein is evident in L4/5 of WT mice (A; 165.7%; $p<0.01$ compared to Naïve; 24h controls used are same as Figure 1). In MMP-9 null mice, TNFa expression is greatly reduced at 24h (48.3%; $p<0.01$ compared to Naïve) but returns to homeostatic levels at 7d (B). MMP-9 null mice do not display compensatory increases in MMP-2 protein at 42d (C), and maintain an acute upregulation of pro-gelatinase MMP-3 at 24h (386.5%Naive; $p<0.05$)

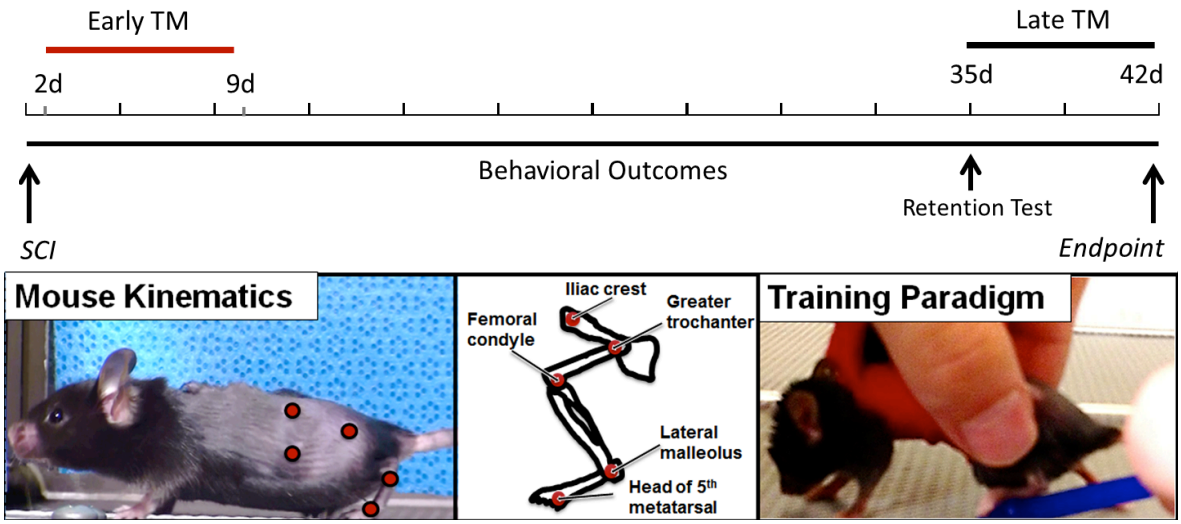

Figure 5. *Mouse kinematics and treadmill training paradigm.* Recovery was examined in two treatment paradigms. Quadrupedal TM training was delivered early (ETM) from 2-9d post SCI, or late (LTM) from 35-42d post SCI. For ETM, recovery was assessed at 7d and retention was examined at 35d. For LTM, recovery was assessed before (35d) and after (42d) the intervention. Bodyweight support was provided using a harness and manual trunk assistance. A pestal was used to ensure plantar placement of steps. Recovery was quantified using two dimensional mouse kinematics. Reflective markers are placed on the iliac crest, greater trochanter, femoral condyle, lateral malleolus, and the head of the 5th metatarsal. Marker tracking was processed using PEAK performance systems.

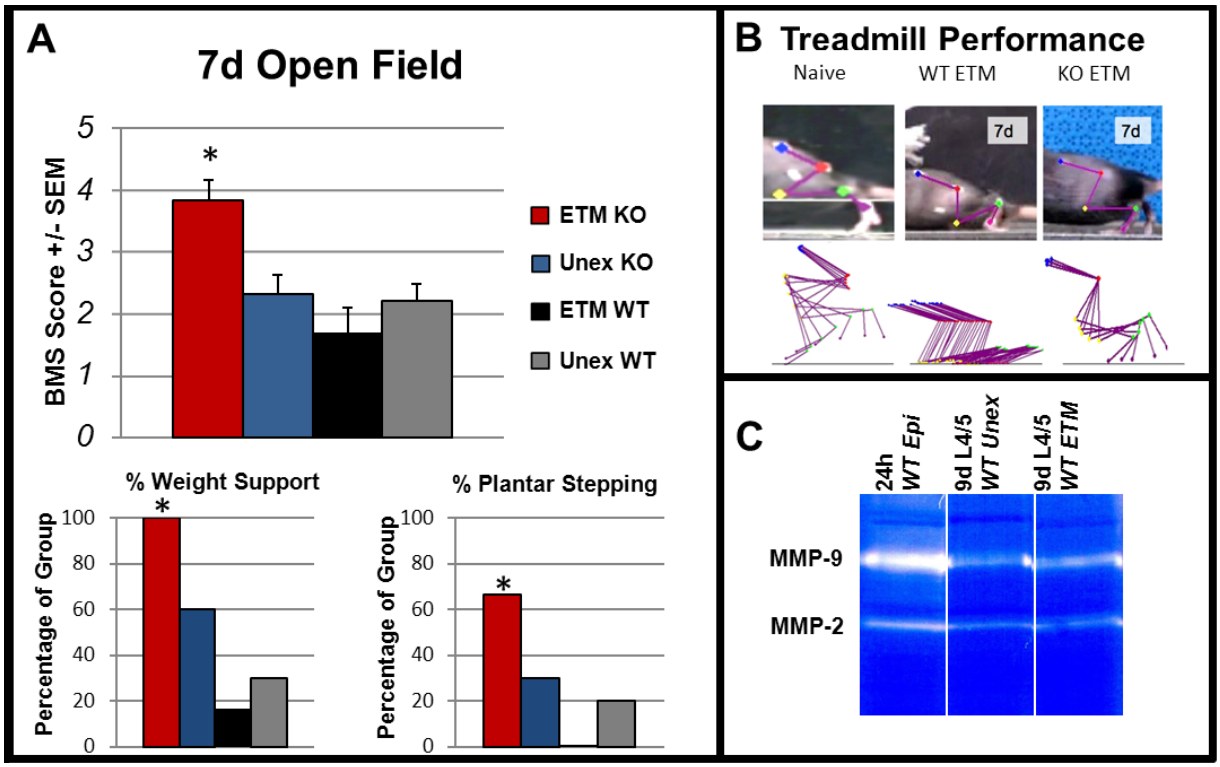

Figure 6. *Robust locomotor improvements occur with early treadmill training in MMP-9 null mice.* Early TM training resulted in significantly better locomotor function in trained MMP-9 null mice. By 7d, trained MMP-9 null mice plantar stepped in the open field (A; BMS=3.83+/-0.32; p<.01). Assessments of weight support and plantar stepping in the open field were significantly higher in the trained MMP-9 null group (Determined using chi square p<0.05). On the treadmill, early trained KO mice independently plantar stepped and no longer required a supportive harness (B). All other groups required bodyweight support and dragged hindlimbs. c) Gelatin zymograms show that MMP-9 expression persists in the lumbar cord (L4/L5) of both trained and untrained WT mice.

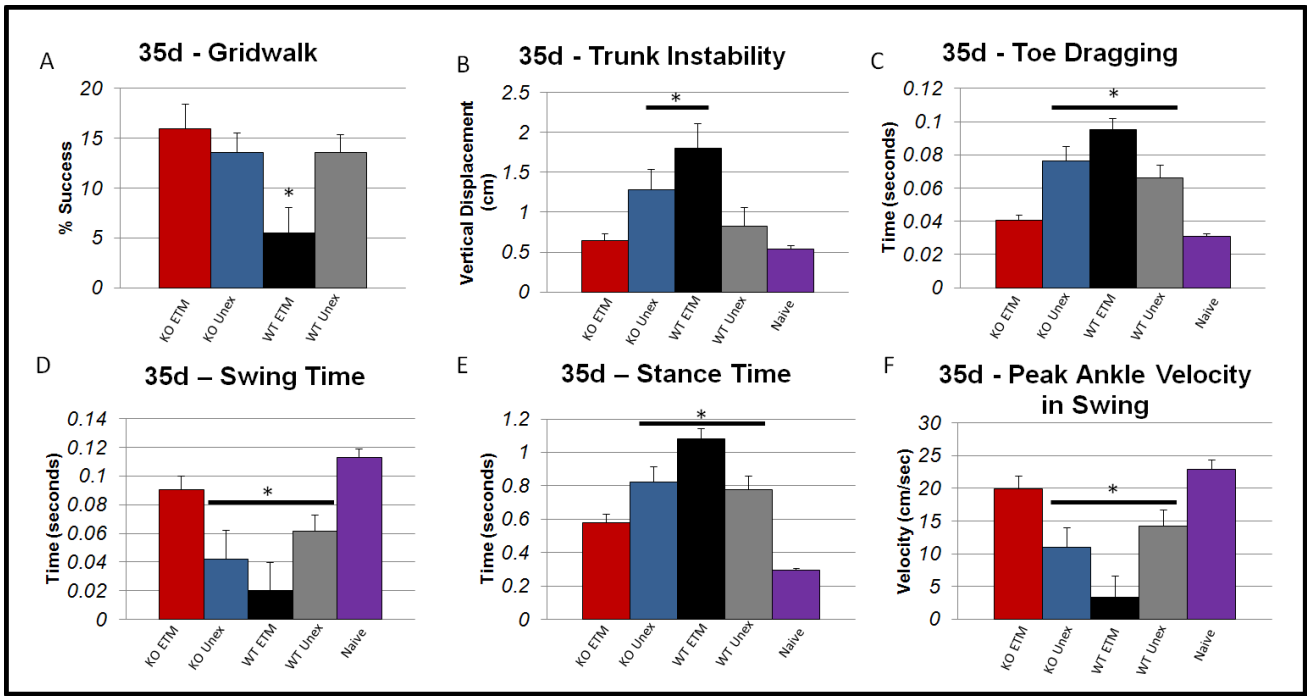

Figure 7. *Retention of Training effects*. Four weeks after training ended (35d), motor control was assessed using gridwalk and TM kinematics. Early training resulted in significantly worse rung-rung paw placement ($p<0.05$) on gridwalk in WT mice (A; Success rate: WT ETM: 5.5%, WT Unex:13.6%, Naïve: 84.4%; $p<0.05$). Early training in MMP-9 null mice (KO ETM) resulted in near-normal measures of trunk instability, toe dragging, time spent in swing/stance, and peak velocity of the ankle during swing (B-E). Trained WT, and untrained controls displayed significant motor deficits compared to naive in all kinematic measures (n=6-10/group; All outcomes compared using MANOVA $p<0.01$).

Reference List

Andrews EM, Richards RJ, Yin FQ, Viapiano MS, Jakeman LB (2012) Alterations in chondroitin sulfate proteoglycan expression occur both at and far from the site of spinal contusion injury. Exp Neurol 235:174-187.

Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23:635-659.

Basso DM, Hansen CN (2011) Biological Basis of Exercise-based Treatments: Spinal Cord Injury. PM R 3:S73-S77.

Basso DM, Murray M, Goldberger ME (1994) Differential recovery of bipedal and overground locomotion following complete spinal cord hemisection in cats. Restor Neurol Neurosci 7:95-110.

Buehner JJ, Forrest GF, Schmidt-Read M, White S, Tansey K, Basso DM (2012) Relationship Between ASIA Examination and Functional Outcomes in the NeuroRecovery Network Locomotor Training Program. Arch Phys Med Rehabil 93:1530-1540.

Busch SA, Horn KP, Silver DJ, Silver J (2009) Overcoming macrophage-mediated axonal dieback following CNS injury. J Neurosci 29:9967-9976.

Buss A, Pech K, Kakulas BA, Martin D, Schoenen J, Noth J, Brook GA (2007) Matrix metalloproteinases and their inhibitors in human traumatic spinal cord injury. BMC Neurology 7:17.

Caudle K, Browh EH, Shum-Siu A, Burke D, Magnuson T, Voor MJ, Magnuson DS (2011) Hindlimb Immobilization in a Wheelchair Alters Functional Recovery Following Contusive Spinal Cord Injury in the Adult Rat. Neurorehabil Neural Repair Epub Ahead of Print.

Cazalets JR, Borde M, Clarac F (1996) The synaptic drive from the spinal locomotor network to motoneurons in the newborn rat. J Neurosci 16:298-306.

Detloff MR, Fisher LC, McGaughy V, Longbrake EE, Popovich PG, Basso DM (2008) Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. Exp Neurol 212:337-347.

Donnelly DJ, Popovich PG (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 209:378-388.

Ethell IM, Ethell DW (2007) Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. J Neurosci Res 85:2813-2823.

Falo MC, Fillmore HL, Reeves TM, Phillips LL (2006) Matrix metalloproteinase-3 expression profile differentiates adaptive and maladaptive synaptic plasticity induced by traumatic brain injury. J Neurosci Res 84:768-781.

Fawcett J (2009) Molecular control of brain plasticity and repair. Prog Brain Res 175:501-509.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, Pasquale-Styles M, Dietrich WD, Weaver LC (2006) The cellular inflammatory response in human spinal cords after injury. Brain 129:3249-3269.

Goslow GE, Jr., Reinking RM, Stuart DG (1973) The cat step cycle: hind limb joint angles and muscle lengths during unrestrained locomotion. J Morphol 141:1-41.

Goussev S, Hsu JY, Lin Y, Tjoa T, Maida N, Werb Z, Noble-Haeusslein LJ (2003) Differential temporal expression of matrix metalloproteinases after spinal cord injury: relationship to revascularization and wound healing. J Neurosurg 99:188-197.

Griesbach GS, Gomez-Pinilla F, Hovda DA (2007) Time window for voluntary exercise-induced increases in hippocampal neuroplasticity molecules after traumatic brain injury is severity dependent. J Neurotrauma 24:1161-1171.

Hains BC, Waxman SG (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. J Neurosci 26:4308-4317.

Hansen CN FLDRSAGJBJaBD (2012) Thoracic SCI results
in lumbar dysregulation of the MMP-axis and a timecourse of locomotor plasticity.

Hansen CN FLDRaBDM (2011) Elevated MMP-9 in the thoracic and lumbar cord
impedes motor learning and recovery early after SCI in mice.

Hansen CN, Linklater W, Santiago R, Fisher LC, Moran S, Buford JA, Basso DM (2012) Characterization of recovered walking patterns and motor control after contusive spinal cord injury in rats. Brain Behav 2:541-552.

Harkema SJ, Schmidt-Read M, Lorenz DJ, Edgerton VR, Behrman AL (2012) Balance and Ambulation Improvements in Individuals With Chronic Incomplete Spinal Cord Injury Using Locomotor Training Based Rehabilitation. Arch Phys Med Rehabil 93:1508-1517.

Hodgson JA, Roy RR, Dobkin B, Edgerton VR (1994) Can the mammalian lumbar spinal cord learn a motor task? Med Sci Sports Exerc 26:1491-1497.

Hoschouer EL, Basso DM, Jakeman LB (2010) Aberrant sensory responses are dependent on lesion severity after spinal cord contusion injury in mice. Pain 148:328-342.

Hougland MT, Harrison BJ, Magnuson DSK, Rouchka EC, Petruska JC (2012) The Transcriptional Response of Neurotrophins and Their Tyrosine Kinase Receptors in Lumbar Sensorimotor Circuits to Spinal Cord Contusion is Affected by Injury Severity and Survival Time. Front Physiol 3:478.

Hsu JY, Bourguignon LYW, Adams CM, Peyrollier K, Zhang H, Fandel T, Cun CL, Werb Z, Noble-Haeusslein LJ (2008) Matrix metalloproteinase-9 facilitates glial scar formation in the injured spinal cord. J Neurosci 28:13467-13477.

Huie JR, Baumbauer KM, Lee KH, Bresnahan JC, Beattie MS, Ferguson AR, Grau JW (2012) Glial tumor necrosis factor alpha (TNF+¦) generates metaplastic inhibition of spinal learning. Plos One 7:e39751.

Jakeman LB, Guan Z, Wei P, Ponnappan R, Dzwonczyk R, Popovich PG, Stokes BT (2000) Traumatic spinal cord injury produced by controlled contusion in mouse. J Neurotrauma 17:299-319.

Jones TA, Schallert T (1994) Use-dependent growth of pyramidal neurons after neocortical damage. J Neurosci 14:2140-2152.

Kawasaki Y, Xu ZZ, Wang X, Park JY, Zhuang ZY, Tan PH, Gao YJ, Roy K, Corfas G, Lo EH, Ji RR (2008) Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. Nature Med 14:331-336.

Kigerl KA, McGaughy VM, Popovich PG (2006) Comparative analysis of lesion development and intraspinal inflammation in four strains of mice following spinal contusion injury. J Comp Neurol 494:578-594.

Kim HJ, Fillmore HL, Reeves TM, Phillips LL (2005a) Elevation of hippocampal MMP-3 expression and activity during trauma-induced synaptogenesis. Exp Neurol 192:60-72.

Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, Joh TH (2005b) Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. J Neurosci 25:3701-3711.

Kloos AD, Fisher LC, Detloff MR, Hassenzahl DL, Basso DM (2005) Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. Exp Neurol 191:251-265.

Kozlowski DA, James DC, Schallert T (1996) Use dependent exaggeration of neuronal injury after unilateral sensorimotor cortex lesions. J Neurosci 16:4776-4786.

Kumar A, Stoica BA, Sabirzhanov B, Burns MP, Faden AI, Loane DJ (2013) Traumatic brain injury in aged animals increases lesion size and chronically alters microglial/macrophage classical and alternative activation states. Neurobiol Aging 34:1397-1411.

Leblond H, L'Esperance M, Orsal D, Rossignol S (2003) Treadmill locomotion in the intact and spinal mouse. J Neurosci 23:11411-11419.

Liu H, Shubayev VI (2011) Matrix metalloproteinase-9 controls proliferation of NG2+ progenitor cells immediately after spinal cord injury. Exp Neurol 231:236-246.

Ma M, Basso DM, Walters P, Stokes BT, Jakeman LB (2001) Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. Exp Neurol 169:239-254.

Ma M, Wei P, Wei T, Ransohoff RM, Jakeman LB (2004) Enhanced axonal growth into a spinal cord contusion injury site in a strain of mouse (129X1/SvJ) with a diminished inflammatory response. J Comp Neurol 474:469-486.

Maldonado MA, Allred RP, Felthauser EL, Jones TA (2008) Motor skill training, but not voluntary exercise, improves skilled reaching after unilateral ischemic lesions of the sensorimotor cortex in rats. Neurorehabil Neural Repair 22:250-261.

McHugh MP (2003) Recent advances in the understanding of the repeated bout effect: the protective effect against muscle damage from a single bout of eccentric exercise. Scandinavian Journal Of Medicine & Science In Sports 13:88-97.

Nesic O, Lee J, Johnson KM, Ye Z, Xu GY, Unabia GC, Wood TG, McAdoo DJ, Westlund KN, Hulsebosch CE, Regino Perez-Polo J (2005) Transcriptional profiling of spinal cord injury-induced central neuropathic pain. J Neurochem 95:998-1014.

Noble LJ, Donovan F, Igarashi T, Goussev S, Werb Z (2002) Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. J Neurosci 22:7526-7535.

Oh LY, Larsen PH, Krekoski CA, Edwards DR, Donovan F, Werb Z, Yong VW (1999) Matrix metalloproteinase-9/gelatinase B is required for process outgrowth by oligodendrocytes. J Neurosci 19:8464-8475.

Parks WC, Wilson CL, L+¦pez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nature Reviews Immunology 4:617-629.

Popovich PG, Horner PJ, Mullin BB, Stokes BT (1996) A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury. Exp Neurol 142:258-275.

Rajashekhar G, Kamocka M, Marin A, Suckow MA, Wolter WR, Badve S, Sanjeevaiah AR, Pumiglia K, Rosen E, Clauss M (2011) Pro-inflammatory angiogenesis is mediated by p38 MAP kinase. Journal Of Cellular Physiology 226:800-808.

Rossignol S, Dubuc R, Gossard JP (2006) Dynamic sensorimotor interactions in locomotion. Physiol Rev 86:89-154.

Smith RR, Brown EH, Shum-Siu A, Whelan A, Burke DA, Benton RL, Magnuson DSK (2009) Swim training initiated acutely after spinal cord injury is ineffective and induces extravasation in and around the epicenter. J Neurotrauma 26:1017-1027.

Vempati P, Karagiannis ED, Popel AS (2007) A biochemical model of matrix metalloproteinase 9 activation and inhibition. J Biol Chem 282:37585-37596.

Vichaya EG, Baumbauer KM, Carcoba LM, Grau JW, Meagher MW (2009) Spinal glia modulate both adaptive and pathological processes. Brain Behav Immun 23:969-976.

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 93:411-422.

Whetstone WD, Hsu JY, Eisenberg M, Werb Z, Noble-Haeusslein LJ (2003) Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. J Neurosci Res 74:227-239.

Yirmiya R, Goshen I (2011) Immune modulation of learning, memory, neural plasticity and neurogenesis. Brain Behav Immun 25:181-213.

Young EE, Baumbauer KM, Elliot A, Joynes RL (2007) Lipopolysaccharide induces a spinal learning deficit that is blocked by IL-1 receptor antagonism. Brain Behav Immun 21:748-757.

Zhang H, Chang M, Hansen C, Basso D, Noble-Haeusslein LJ (2011) Role of Matrix Metalloproteinases and Therapeutic Benefits of Their Inhibition in Spinal Cord Injury. Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics Epub ahead of print.